Nano-Porous Silicon Based Immune Biosensor for the Control of Level of Mycotoxins

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Abstract

The efficiency of the nano-porous silicon (sNPS) application as the transducer in the immune biosensor designed for the level control of mycotoxins among environmental objects was studied. T2 and patulin were chosen as model objects. Boron doped single-crystal silicon square wafers with the resistivity of 1 Ohm·cm, area of 100 cm2 and thickness of 0.3 μm was used. The surface was prepared by stain etching in 4HF:1HNO3:4H2O solution. sNPS surface is regularly covered with nano-scale hills up to 20 nm high. The registration of the specific signal was made on the basis of changes of chemiluminescence (ChL) or photocurrent of the sNPS. The biosensor sensitivity for both variants was 10-20 ng/ml at the total time of analysis lasting for 40 min. This time may be a sharp decline if specific antibodies (Ab) will be preliminary immobilized. It was concluded that the proposed immune biosensor was effective if the analysis will be fulfilled in screening regime. For the verification of the results or for more accurately determination of mycotoxin level, it is necessary to find way for increasing sensitivity, or to apply another analytical approach.

Keywords

Nano-structured Silicone, Immune Biosensors, T2 Mycotoxin, Patulin, Determination.

Introduction

Mycotoxins presented by T2, aflatoxins, searelenone, patulin and others cause a grate interest since they are widespread and characterized with high level toxicity. The main attention for food administration in all countries is given to T2-mycotoxin as most toxic and wide dispersed substance from one side and as representative of biological weapons and object of bioterrorist interests from other one [Starodub, 2009; 2012).

Patulin (4-hydroxy-4H-furo [3,2c] pyran-2[6H]-one) produced by a number of strains of fungies: Penicillium, Aspergillus, Byssochlamys and Paecilomyces originally was discovered as an antibiotic [Stott, Bullerman, 1975; Ciegler, 1977]. The most common producer of it is Penicillium expansum. This mycotoxin is affected by rot in many vegetables, fruits and cereals. But apples and their products are its most characteristic source. In spite of existing data about the carcinogenic properties patulin was referred to 3-d group of toxic substances according to International cancer agency since its such effects were not be exact established [IECFA, 19960]. Nevertheless, it was classified as immune depressant, mutagen and cytotoxic agent as well [Sherif at al., 2009; Appell et al., 2009; Songül at al., 2011]. Since 1995, several countries have introduced rationing patulin content, mainly in fruits and products of their processing. In most countries, it is established beyond a content patulin at the level of 50 μg/kg and less [Moake et al., 2005; FAO, 2003]. Patulin is especial dangerous for babies [Songül et al., 2011].

Unfortunately, the analytical methodologies for analysis of mycotoxins as well as other low molecular weight toxins include such instrumental approaches as liquid or gas chromatography with mass spectroscopy and their high-performance variants [Nosenko et al., 2009; Starodub et al., 2011). Due to the extremely high complication and cost of analysis fulfilled by these methods, the development of innovative approaches, such as immune analysis and particular chemo- and biosensors, is very urgent [Starodub, 2009, 2012]. Early [Nabok et al., 2007; 2007a; 2011; Starodub et al., 2011] a number of types of optical immune biosensors have been developed based on the surface plasmon resonance and total internal reflection ellipsometry (TIRE) as well as some electrochemical ones.

With the discovery of the enhanced photo- and electroluminescence of sNPS [Uhlir, 1956; Canham, 1990] numerous investigations have been undertaken

to study these effects and to utilize them at the creation of optoelectronic devices such as light emitting devices, gas sensors, photodetectors and solar cells [Turner,1989; Starodub et al., 2000a]. Being a promising material for the device technology, sNPS also excites great interest among scientists working with biosensors with the purpose of quick detection of a biological substance and in small quantities. This is critically important for the express diagnostics of diseases and environmental monitoring. Several methods have been proposed to obtain PS and to use its properties at the creation of different devices [Starodub at al., 1996; 2000; Becker et al., 2009; Sailor, 2012].

To fulfill all practice demands in respect of high sensitivity of analysis as well as simplicity, cheapness and rapidity of its fulfillment at the control of mycotoxins, sNPS was put forward to be employed as transducers for the immune biosensors with the registration of the specific signal on the basis of changes of ChL or photocurrent of this structure. The information concerning investigations on some physical-chemical abilities of sNPS, worked out algorithm of analysis, obtained results and possible mechanism of specific signal formation is main goal of this report. As model of low molecular weight toxins we used T-2 mycotoxin and patulin.

Experimental

Boron doped single-crystal silicon square wafers with resistivity of 1 Ohm·cm, with area of 100 cm2 and thickness of 0.3 μm from "Microprocessor" (Ukraine) was in use. The surface of the wafers was not polished. sNPS layers were prepared by stain etching in the solution of HF: HNO3: H2O in the ratio of 4:1:4 at the room temperature, natural day-time illumination and time duration from 1 to 20 min. Thickness of sNPS layer changing from 3 up to 60 nm, was supervised by parameters of technological process at chemical modification of a surface of single-crystal silicon and defined with the help of Auger electronic spectroscopy at the LAS-2000. The structure of sNPS surface was studied using scanning tunnel microscope (STM) and scanning electron microscope (SEM). Analysis on the obtained images of the surface showed that the sNPS surface is regularly covered with nano-scale hills up to 20 nm high (Fig. 1). The scheme of the optical device based on the sNPS for the photo resistance registration of the signal at the formation of the specific immune complex is given in Fig. 2. The detailed explanation of the used contacts and layers in the immune biosensor based on the sNPS is presented in Fig. 3. At the beginning of the measurement, the specific Ab in the volume of 1μl was placed on the photoresistor surface between the contacts. Then this solution was evaporated at the room temperature or at the air stream. The direct voltage (5 V) from the stabilized power supply was applied to the ohmic contacts and the current was measured by the digital voltmeter of B7-35 type at the absence of lighting (dark regime) as well as the photocurrent (the difference between the light and dark currents) was registered at the lightening of the sensitive surface by the white spectrum light (lamp with the illumination of 7000 lux, "Microprocessor", Ukraine). At the drawing of antigen layer on the sensitive plate and after its drying, the measurements of the dark and light current were repeated. They were made after the immune complex formation (interaction of antigen with the specific Ab in the serum blood) as well. The control of the reaching of the sensor initial state was done according to the reduction of the dark current value after washing the sensitive surface by the buffer solution. The time of the single analysis was 5-10 min only.

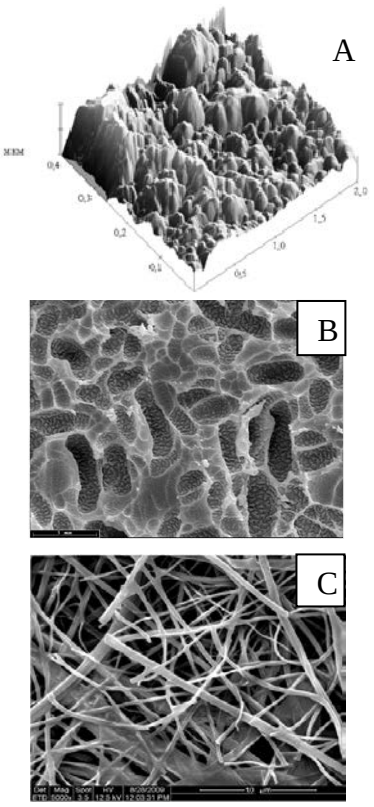

FIG. 1 STM (A) AND SEM (B AND C) IMAGES OF THE sNPS SURFACE (A,B) AND SiO2 NANOTUBE (C)

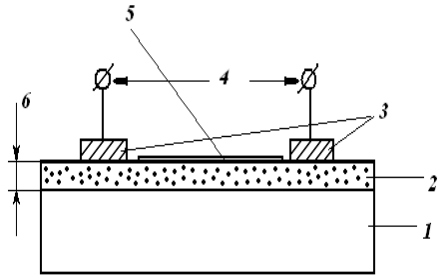

FIG. 2 PHOTORESISTOR STRUCTURE BASED ON THE sNPS. 1 – CRYSTALLINE SILICON, 2 – sNPS, 3 – ELECTRICAL CONTACTS, 4 – APPLIDE VOLTAGE, 5 – BIOLOGICAL OBJECT, 6 – THICKNESS OF THE sNPS

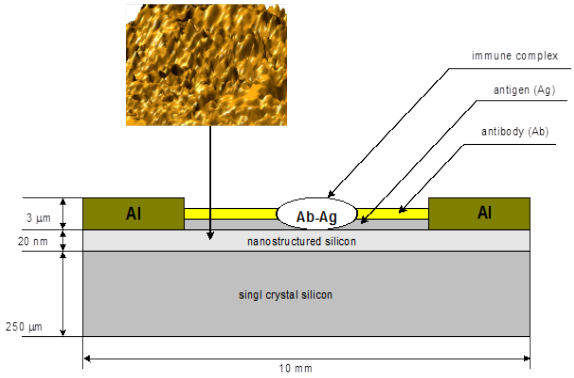

FIG. 3 DETAILED CHARACTERISTICS OF THE PHOTORESISTOR STRUCTURE BASED ON THE sNPS WITH THE CONCRETIZATION OF THE DIMENSIONS OF CONSTRUCTION

Design of the prototype for the registration of the specific immune complex by the PhL of the sNPS includes the source of the ultraviolet (UV) radiation with the wavelength of 350 nm, two photodiodes (2 and 3) based on the mono crystalline silicon and placed at the angle of 20-250 relatively to the plate with the layer of the sNPS and the photo diode intended for the determination of the incident UV (Fig. 4). Application of two photo registers of the PhL increases the sensitivity. To avoid the possible changing of the incident UV, the additional photodiode is used. Photodiodes of the n-p-p+-structures work in the photo generative regime. Such construction is related to the systems of the differential type. Photo diodes were obtained from "Microprocessor", Ukraine. At the adsorption of the biological molecules, the level of the PhL of the sNPS and the output of the voltage of the consecutive connected photo registers are decreased.

The immune biosensor analysis was fulfilled in "direct" way when specific antibodies (Ab) were immobilized on the sNPS surface and then reacted with appropriate mycotoxin in model solution.

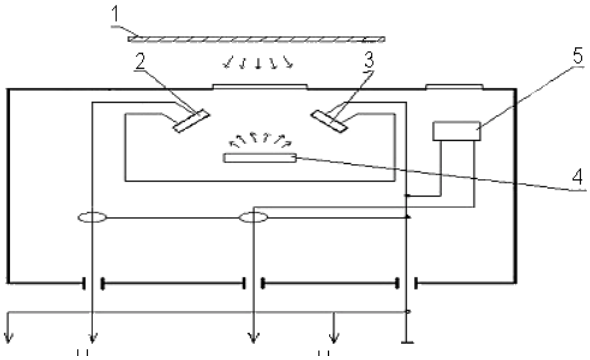

FIG. 4 PhL BIOSENSOR, WHERE: 1 – SOURCE OF THE ULTRAVIOLET (UV) WITH λ AT 350 nm, 2 AND 3 – TWO SLIDES OF THE sNPS, 4 – PHOTO DIODE, 5 – PHOTO DIODE FOR THE CONTROL OF THE INSIDED UV

To examine the efficiency of the proposed immune biosensor in case of the analysis of real products, oatmeal as well as tomato and pomegranate juices were selected as model objects taking into account that fruits and vegetables are predominantly affected by both mycotoxins and, especially, by patulin. Extraction procedure is performed as follows. To tomato paste, or 1 g of oatmeal, or 5 ml juice (pomegranate or tomato) soaked in the solvent in a ratio of 1:1 were added mycotoxin solution with careful stirring and its overall final concentration was kept at 200 ng/ml. After 2 hours exposure of samples in sealed conditions, another half solvent it was added and kept for 2 h. Then aliquot was taken by passing mixture through a filter paper and at last the content of mycotoxin was determined.

At the all steps of the study on the efficiency of the application of the developed immune biosensors, the ELISA-method served as control over the level of the specific and non-specific immune reaction. This method was fulfilled based on conventional approach [Tutel'jan et al., 1984; Ngo, Lenhoff, 1985].

The synthesis of conjugates of patulin with horse radish peroxidase (HRP) was fulfilled in several steps according to scheme described previously (de Champdore et al., 2007]. Patulin as well as T2 mycotoxin and specific Ab to the last were obtained from "Sigma-Oldrich" (USA). Specific Ab to patulin was produced by "Agrisera" (Sweeden). A conjugate of T2 with bovine serum albumin was prepared according to [Burkin and Kononenko, 2004].

Results and Discussion

Photoelectric processes in the layers of the sNPS which belong to the semiconductors materials have been accomplished in the result of the photo generation of the electron-holes pairs and following

their dividing and recombination. The processes of adsorption on the sNPS surface may arouse new photoelectric effects. Nanocrystallites of the silicon with the dimensions from one to dozen nm are as the silicon regions which are not dissolved and surrounded by the production of the electrochemical and the chemical reactions. At the dimensions less than 15-20 nm, the quant-dimensioned effects have been aroused which lead to the quantization of the energetic spectra of the charge curriers, the widening of the prohibited zone up to 1.7-3.4 eV and to the decrement of the dielectric permeability. The lux-ampere characteristics of the obtained samples have two plots: the linear and the sub linear which achieves the saturation at the illumination more than 10000 lux. The samples with the nanolayer thickness of 15-18 nm have the maximal photosensitivity (Fig. 5).

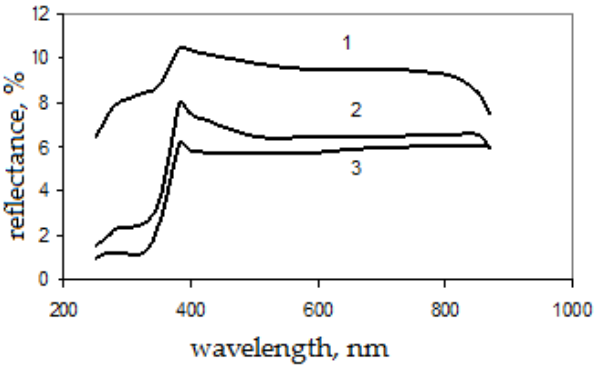

FIG. 5 SPECTRA OF THE OPTICAL REFLECTANCE OF THE SNPS LAYER IN THE DEPENDENCE ON THE DIMENSION OF NANOCRYSTALLITES: 1 – 5 NM, 2 – 15 NM, 3 – 30 NM

The maximal photosensitivity had a good correlation with the results of the experiments with the PhL (Fig. 6).

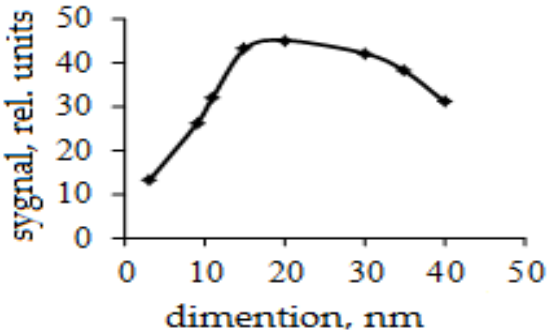

FIG. 6 DEPENDENCE OF THE INTENSITY OF THE PHL OF THE SNPS LAYERS AT THE 650 NM

It is necessary to mention that the changing of the etching content and the solution concentrations brings to the change of the dynamics of NPS layer growth, the porosity level, the correlation of the dimensions of the crystallites and the holes, the chemical content and the profile of the dispersion of main admixtures.

Our investigations on the sNPS showed that the samples prepared by the method of the chemical etching have stable ChL, conductivity and photoconductivity characteristics which were preserved during several years.

As a rule, in the development of the immune biosensors based on the surface plasmon resonance and total internal reflection ellipsometry to achieve high density of the immobilization of the immune components on the transducer surfaces, they were preliminary treated with one of some chemical substances among of which the most used are: a) dextran sulphate; b) dodecanthiol; c) polyelectrolytes: polyalylamine hydrochloride or/and polystyrene sulphat [Starodub, 2009; 2011; 2012]. After that, the transducer surface was treated with some substances to achieve oriented immobilization of specific antibodies in advance, among which the most applied are: a) protein A from Staphylococcus aureus; b) protein G from Staphylococcus; c) lectins. As a rule in the ELISA-method and in the most cases at the application of the immune biosensors, the different regimes of analysis are used. For example, "competitive" way when immobilized hapten is conjugated with some protein competes with free one for the specific Ab. Other variant of such analysis may be realized if the free and conjugated hapten competes for the specific Ab immobilized on the transducer surface. Unfortunately, during the development immune biosensors based on the sPNS, we realized very simple "direct" way of analysis only when specific Ab is immobilized on the transducer surface interacting with free hapten. It is connected with some problem of the immobilization of components on the sNPS surface and their influence on the formed signal. These effects are planned to be investigated in details in future studies.

The detailed results of mycotoxins analysis are presented in Fig. 7-9. It is shown that the sensitivity of the proposed immune biosensor for both ways of the specific signal registration (by the ChL and electroconducticity) allows determining T-2 mycotoxin and patulin at the concentration of 10 ng/ml during several minutes.

To compare results of the proposed immune biosensor analysis with some traditional immune testing, we obtain results of the determination of T2-mycotoxin and patulin by means of the standard ELISA-method. The level of the sensitivity of this method at the determination of both mycotoxins in

the case "competitive" analysis has been examined.

It was carried out by two ways as it was mentioned in the experimental part of this article. It was stated that ordinarily the level of the sensitivity of the analysis for both mycotoxins was in frame of 10 ng per ml with the linearity up to about 10 μg per ml. As an example of such analysis the curve of dependence of extinctions of cells of immunological plates on the concentration of T2 mycotoxin is given in Fig. 10.

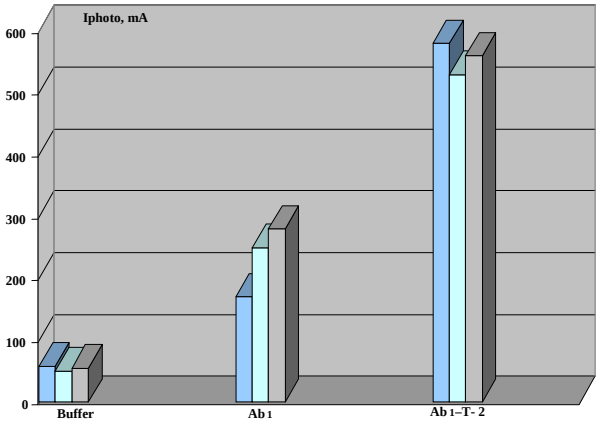

Fig. 7 CHANGES OF PHOTOCURRENT OF PHOTORESISTOR AFTER LOADING OF BUFFER, SPECIFIC ANTIBODIES (Ab_1) AND FORMATION OF Ab_1T-2 MYCOTOXIN COMPLEX

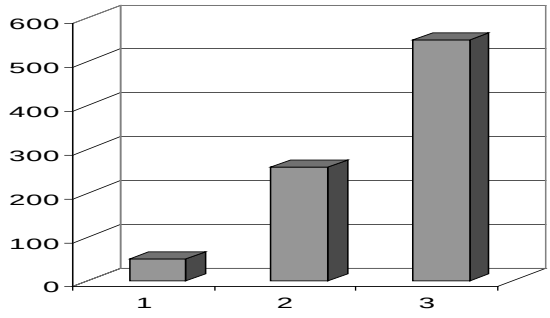

FIG. 8 DEPENDENCE OF sNPS PHOTOCURRENT AT THE SURFACE STATE: 1 – BARE; 2 – WITH Ab AND 3 – WITH THE SPECIFIC Ab AND PATULIN

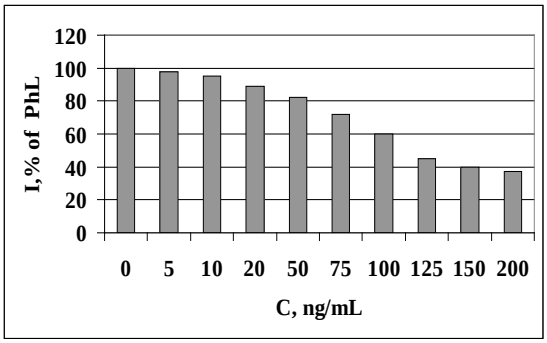

FIG. 9 DEPENDENCE OF THE IMMUNE BIOSENSOR SIGNAL (INTENSITY OF sNPS PhL) ON THE CONCENTRATION OF T2-MYCOTOXIN IN THE SOLUTION TO BE ANALYZED

The total duration of the fulfillment of all steps of the ELISA-method is about 6 hours. In case of the proposed immune biosensor analysis, the whole spent time including Ab immobilization and all steps of measurements is about 40 min. This time may be shortened if Ab will be immobilized preliminary and analysis will be started beginning with the mycotoxin loading on the sPNS surface. The obtained calibration curves with the model solution of T-2 mycotoxin and patulin open perspective for the practical application of the proposed immune biosensor in case of the determination of other micotoxins as well as other types of toxic substances with the use of their specific Ab.

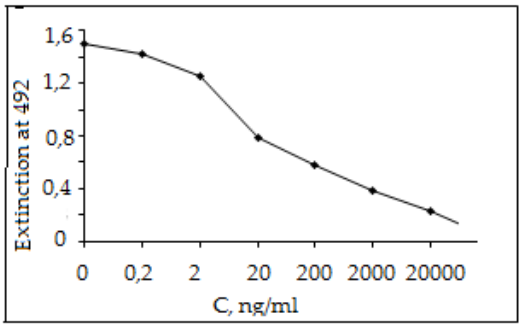

FIG. 10 CURVE OF THE T2 MYCOTOXIN DETERMINATION BY ELISA-METHOD

Based on our opinion, the red PhL may be connected with the tunnel mechanism of the recombination of the charge bearers at the excitation of them in the nano-crystallites of oxide or interface (Koch, 1993). We do not exclude hydrogen role too for the generation of the PhL extinguishing (Starodub et al., 1996. These conclusions are as result of the coincidence of the possible reasons for the PhL decreasing in case of the immune complex formation on the sNPS surface. These reasons include: a) the changes of the absorbance in the solution at the formation of the specific immune complex on the sNPS surface, b) the effect of the immune components or their interaction on the recombinant process of the photocurrent charge in the sNPS. As it is very known the light absorption in the wavelength of the excitation (350 нм) and in the wide field of the sNPS PhL is absent in the Ab and antigen solutions as well as in their complexes. Of course, it is necessary to understand what are the factors influencing the primary immune components (in this case of the specific antibodies) on the process of the recombination in the sPNS, which will be our next task of investigation.

In order to further test efficiency of sNPS as transducer of immune biosensor at the some

mycotoxin determination, investigation was carried out with the real samples. But it preliminary studied what is the best way for the extraction of mycotoxins from the samples to be analyzed. According to the data literature (Nosenko et al., 2009; Starodub et al., 2010) for this purpose, it is necessary to use solvents with some hydrophobic properties. Among two chosen solvents (methanol and acetonitrile), the best results were obtained for the last than first one in the respect of effects on the immune chemical reaction from one side and for the intensity of process of extraction from other ones. In the course of the experiments, it was found that it is possible to detect about 62-79% of T2 mycotoxin if its extraction from pomegranate juice will be made by methanol and up to 90% when acetonitrile will be used for this purpose (Table 1).

It should be noted that the methanol in the sample to 20%, and acetonitrile up to 30% at a subsequent dilution in two times of the initial extraction solution did not affect the intensity of immune-chemical reaction (Table 2).

It is necessary to underline that the level of this reaction at the absent of solvent in the analyzed sample was taken as maximum possible value equal to 100% and other indexes were calculated with respect to a given level.

Of course, not all quantity of mycotoxins can be extracted from a particular product. It is understandable why, namely, some of their quantities are irreversibly sorbet on small particles of vegetable tissue. And the amount of mycotoxin which remains in the mass of the product after extraction depends on its nature and contents of included components.

In this case, the output of micotoxin was on some percent of lower. It is required to build a special calibration curves for each individual product.

TABLE 1 EFFICIENCY RECOVERING OF T2 AT THE EXTRACTION FROM OAT AND TOMATO JUICE BY TWO DIFFERENT SOLVENTS

N	Type of solvents	Type of product	% of mycotoxin reccovery
1	Methanol	Oat	62±3,0
2		Tomato juice	70±3,5
3		Pomegranate juice	79±2,5
4	Acetonitrile	Oat	81±4,1
5		Tomato juice	88±3,2
6		Pomegranate juice	95±2,1

TABLE 2 INTENSITY OF IMMUNOCHEMICAL REACTION (%) AT THE PRESENCE OF METHANOL OR ACETONYTRILE IN PATULIN SAMPLE

Concentration solvent in sample to be analyzed, %	Type of solvent and intensity of immune chemical reaction	
	Acetonitrile	Methanol
1	100	100
10	100	100
20	99	97
30	97	89
40	80	70

On the next step of investigations, the efficiencies of the determination of T2 mycotoxin in the number of products was compared by two different optical immune biosensors based on the TIRE and sNPS. These comparative results for both these biosensors are presented in Table 3. The results about the sensitivity of TIRE based immune biosensor were published early [Nabok at al., 2007; 2007a]. Moreover, to appreciate efficiency of the proposed biosensor, its sensitivity has been in comparison with that inherent to other similar approaches including the ELISA-method as well (Table. 4).

TABLE 3 COMPARISON OF THE EFFICIENCY OF THE DETERMINATION OF T2-MYCOTOXIN LEVEL IN DIFFERENT SOURCES BY THE IMMUNE BIOSENSORS BASED BASED ON THE ELLIPSOMETRY AND SNPS

N	Type of product	Level of T2 mycotoxin content (ng/ml) determined by immune biosensors based on:	
		elipsometry	sNPS
1	Mold hay	>600	~200
2	Mold bread	>600	~250
3	Mold mays	>600	~220
4	Mold buckwheat	>600	~250
5	Stale bread	<1,5	none
6	Stale muesli	<1,5	none
7	Stale buckwheat	7,5-15	traces
8	Fresh muesli	none	none

These approaches have been chosen from two points of view, the first of which was connected with very high sensitivity of TIRE based biosensor in our previous investigations, while the second is stipulated that the algorithm of work for both mentioned immune biosensors should include “direct” way of analysis. It can be seen that the immune biosensor based on the sNPS is much less sensitive than that used TIRE as registering part. However, the first immune biosensor may provide very simple analysis. That it is why the immune biosensor based on the sNPS can serve for screening inspections to inform us about the critical (official permissible) level of mycotoxins in products other one (used TIRE as transducer) may be applied to the verification of the results of analysis. This conclusion confirms data from Table 4 concerning the sensitivity of different methods

of analysis on mycotoxins, including different types of the immune biosensor which have been early proposed (Nabok et al., 2007; 2007a; Starodub et al., 2006).

TABLE 4 COMPAROSIN OF THE SENSITIVITY OF THE DIFFERENT IMMUNE BIOSE3NSORS AT THE CONTROL OF T2 IN SOLUTION TO BE ANALYZED

N	Type of the immune biosensor based on:	Sensitivity	References
1	TIRE	0,15 ng/ml	(Nabok et al., 2007)
2	SPR and thermistors ("direct" analysis)	~ 1,0 μg/ml	(Starodub et al., 2006)
3	SPR ("to saturated" analysis)	~5,0-10,0 ng/m	(Starodub et al., 2006)
4	Piesocrystal ("competitive" analysis)	1,5 ng/ml	(Nabok et al., 2007)
5	sNPS, ChL ("direct" analysis)	20 ng/ml	This article
6	sNPS, photocurrent, ("direct" analysis)	~10 ng/ml	''-''
7	ELISA-method ("competitive" analysis)	~10 ng/ml	''-''

Conclusion

It was demonstrated that porous silicon may be as very effective transducer for immune biosensor which can be used as analytical devices for the determination of such wide spreading toxins as T-2 mycotoxin and patulin. To register the generated specific signal, it is possible to determine such parameters as level of changes of PhL or photocurrent of this structure. Unfortunately, the sensitivity of the device based on this structure is not much higher level but it allows providing screening inspections of environmental objects on the presence toxic substances. A good efficiency of work of the immune biosensor has been shown based on the sNPS but not at the analysis of the model solution of mycotoxins and in case revealing their concentration in the real samples. Due to the maximal sensitivity of the sNPS, based immune biosensor is on the level of permissible concentration of mycotoxins in foodstuffs, for the verification of the results of analysis, namely to have more accurate determination of their level, it is necessary to find way to increase this index for the proposed biosensor, or to apply another analytical approach. By means of taking into account possible practical application of the proposed immune biosensor, it was demonstrated that the use of acetonitrile for the extraction of micotoxins is more effective than methanol and first one may be applied as basic solvent for the preliminary preparation of the samples for the analysis by this analytical approach.

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